

Product Information

TUMOR NECROSIS FACTOR- α , HUMAN ELISA KIT

For quantitative determination of TNF- α

Product Number **CKH-200A**

Storage Temperature 2-8 °C

TECHNICAL BULLETIN

Product Description

Principle of the Assay

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for TNF- α has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and the immobilized antibody binds any TNF- α present. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for TNF- α is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of TNF- α bound in the initial step. The color development is stopped and the intensity of the color is measured.

Current bioassays used for the detection of TNF- α are usually based on the cytolytic effects of TNF- α on responsive cell lines, such as L929. These bioassays are tedious and are not specific for human TNF- α . This TNF- α assay is a 3.5 - 4.5 hour solid phase ELISA that is designed to measure TNF- α in cell culture supernatants, serum, and plasma. It contains *E. coli*-derived recombinant human TNF- α and antibodies raised against this protein. It has been shown to accurately quantitate recombinant human TNF- α . Results obtained on natural TNF- α samples showed linear curves that were parallel to the standard curves obtained using the kit standards. These results indicate that this TNF- α assay can be used to determine relative mass values for natural TNF- α . The measurement of TNF- α is insensitive to the addition of recombinant forms of either of the two types of soluble receptors. Therefore, it is probable that this measurement detects the total amount of TNF- α in samples, i.e., the total amount of free TNF- α plus the amount of TNF- α bound to soluble receptors.

Background

Tumor necrosis factor alpha (TNF- α),^{1,2} also known as cachectin, and tumor necrosis factor beta (TNF- β),^{3,4} also known as lymphotoxin, are two closely related proteins (about 34% amino acid residue homology). They bind to the same cell surface receptors and produce a vast range of similar, but not identical, effects. In contrast to the similarity of their biological activities, the regulation of the expression and processing of the two factors is quite different.^{5,6} TNF- α is produced by neutrophils, activated T and B lymphocytes, NK cells, LAK cells, astrocytes, endothelial cells, smooth muscle cells, and some transformed cells.^{5,6} TNF- β is produced by lymphocytes.^{5,6} The properties and activities of the TNFs have been the subject of numerous reviews.⁵⁻¹¹

Mature human TNF- α is a polypeptide of 157 amino acid residues (mouse, rat, and rabbit TNF- α are one amino acid shorter).⁵ The apparent molecular weight of human TNF- α under denaturing conditions is approximately 17 kDa.¹² Human TNF- α , in contrast to TNF- β , shows no N-glycosylation (mouse TNF- α is N-glycosylated).⁵ The biologically active native forms of both TNF- α and TNF- β are trimers.^{13,14}

TNF- α , unlike TNF- β , does not possess a typical signal peptide sequence. TNF- α is, however, initially synthesized as a larger protein with the mature 17 kDa factor comprising the C-terminal portion of this precursor. The N-terminal sequence of the precursor contains both hydrophilic and hydrophobic domains and its presence results in the occurrence of TNF- α as a membrane-bound form from which the mature factor is released by proteolytic cleavage.¹⁵⁻¹⁷ Evidence suggests that the membrane-anchored form of TNF- α on the surface of macrophages and/or monocytes, in addition to serving as a reservoir for release of soluble TNF- α , has lytic activity and may also have an important role in intercellular communication.¹⁵⁻¹⁷

Two distinct receptor types have been identified that specifically bind TNF- α and TNF- β . Virtually all cell types studied show the presence of one or both of

these receptor types. One type, TNFR-II (Type A, Type α , 75 kDa or utr antigen), is a transmembrane glycoprotein with an apparent molecular weight of 75 kDa.¹⁸ The other type, TNFR-I (Type B, Type β , 55 kDa or htr antigen), is a transmembrane glycoprotein with an apparent molecular weight of 55 kDa.^{19,20} The two receptor types are distinct immunologically, but show similarities to each other and to the NGF receptor in the pattern of cysteine residue locations in four domains in their extracellular portions.^{5,18} The intracellular domains of the two TNF receptor types are apparently unrelated, suggesting that the two receptor types employ different signal transduction pathways.¹⁸ Each receptor type can bind TNF- α or TNF- β with high affinity and there is no evidence that interaction between the two receptor types is necessary for signal transduction.²⁰⁻²² Soluble forms of both types of receptors have been found in human serum and urine.²³⁻²⁵ These soluble receptors are capable of neutralizing the biological activities of both TNF- α and TNF- β and may serve to modulate and localize the activities of the TNFs or may serve as a reservoir for the controlled release of the TNFs.

The two TNFs are extremely pleiotropic factors. The ability to produce a wide variety of effects is attributed to the ubiquity of their receptors, to their ability to activate multiple signal transduction pathways, and to their ability to induce or suppress the expression of a vast number of genes. These genes include those for growth factors and cytokines, transcription factors, receptors, inflammatory mediators and acute phase proteins, etc.^{5,26} TNFs play a critical role in normal host resistance to infections and to the growth of malignant tumors, serving as immunostimulants and as mediators of the inflammatory response. Many of the actions produced by the TNFs are functionally similar to the effects produced by IL-1.

On the other hand, over-production of TNF has been implicated as playing a role in a number of pathological conditions, including cachexia (progressive wasting),^{2,27} septic shock following infection with gram-negative bacteria,²⁸ autoimmune disorders,²⁹ and meningococcal septicemia.³⁰ Two studies have found elevated levels of TNF- α in the cerebrospinal fluid (CSF) of multiple sclerosis (MS) patients, particularly those with active rather than stable disease.^{31,32} TNF- α was also detected histologically in MS lesions.³³

Components

1. **TNF-a Microplate** **T 8424**
(1 each)
96 well polystyrene microplate (12 strips of 8 wells) coated with a murine monoclonal antibody against TNF- α .
2. **TNF-a Conjugate** **T 8549**
(1 vial, 21 ml)
Polyclonal antibody against TNF- α conjugated to horseradish peroxidase, with preservative.
3. **TNF-a Standard** **T 8674**
(3 vials, 1 ng/vial)
Lyophilized recombinant human TNF- α in a buffered protein base with preservative.
4. **Assay Diluent 1F** **D 1550**
(1 vial, 6 ml)
Buffered protein base with preservative. Contains a precipitate.
5. **Calibrator Diluent 5** **D 1300**
(1 vial, 21 ml)
Buffered protein base with preservative. *For cell culture supernatant testing.*
6. **Calibrator Diluent 6** **D 4300**
(1 vial, 21 ml)
Animal serum with preservative. *For serum/plasma testing only.*
7. **Wash Buffer Concentrate** **W 4379**
(1 vial, 21 ml)
25-fold concentrated solution of buffered surfactant with preservative.
8. **Color Reagent A** **R 6517**
(1 vial, 12.5 ml)
Stabilized hydrogen peroxide.
9. **Color Reagent B** **R 6642**
(1 vial, 12.5 ml)
Stabilized chromogen (tetramethylbenzidine).
10. **Stop Solution** **S 0416**
(1 vial, 6 ml)
2 N sulfuric acid.
11. **Plate Covers** **C 5697**
(4 each)
Adhesive strips.

Other Supplies Required but not supplied

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Multi-channel pipette, squirt bottle, manifold dispenser, or automated microplate washer.
- 500 mL graduated cylinder.

Precautions and Disclaimer

Some components of this kit contain sodium azide which may react with lead and copper plumbing to form explosive metallic azides. Flush with large volumes of water during disposal.

The Stop Solution provided with this kit is an acid solution. Wear eye, hand, face, and clothing protection when using this material.

This kit contains thimerosal, a mercury containing compound. The total amount of mercury in this kit is 13.3 mg.

Preparation Instructions

Sample Collection and Storage

Cell Culture Supernatants

Centrifuge to remove particulate material and assay immediately or aliquot and store samples at -20°C . Avoid repeated freeze-thaw cycles.

Serum

Use a serum separator tube (SST) and allow samples to clot for 30 minutes before centrifugation for 10 minutes at approximately $1,000 \times g$. Remove serum and assay immediately or aliquot and store samples at -20°C . Avoid repeated freeze-thaw cycles.

Plasma

Collect plasma using EDTA, heparin, or citrate as an anticoagulant. Centrifuge at $1,000 \times g$ within 30 minutes of collection. Assay immediately or aliquot and store samples at -20°C . Avoid repeated freeze-thaw cycles.

Caution: If using citrate or heparin collection tubes, make sure they are pyrogen-free to avoid endogenous TNF- α production.

Reagent Preparation

Bring all reagents to room temperature before use.

Wash Buffer

If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 20 ml of Wash Buffer Concentrate into deionized or distilled water to prepare 500 ml of Wash Buffer.

Substrate Solution

Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. 200 μl of the resultant mixture is required per well.

TNF- α Standard

Reconstitute the TNF- α Standard (T 8674) with 1 ml of deionized water (for cell culture supernatant samples) or Calibrator Diluent 6 (D 4300) for serum/plasma samples, or a diluent that reflects the composition of the samples being assayed. This reconstitution produces a stock solution of 1,000 pg/ml. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions. Use this stock solution to produce a dilution series, as described below, within the range of this assay (15.6 pg/ml to 1,000 pg/ml). Further dilute the standard as necessary.

A suggested dilution series for standards is

1,000 pg/ml, 500 pg/ml, 250 pg/ml, 125 pg/ml, 62.5 pg/ml, 31.3, pg/ml, 15.6 pg/ml and 0 pg/ml.

- Label six tubes: 500 pg/ml, 250 pg/ml, 125 pg/ml, 62.5 pg/ml, 31.3, pg/ml, and 15.6 pg/ml.
- Pipette 500 μl of the appropriate diluent (Calibrator Diluent 5 for cell culture supernatant samples or Calibrator Diluent 6 for serum/plasma samples) into each tube.
- Pipette 500 μl of the 1,000 pg/ml TNF- α standard stock solution into the 500 pg/ml tube and mix thoroughly.
- Pipette 500 μl of the 500 pg/ml standard solution into the 250 pg/ml tube and mix thoroughly.
- Repeat the process successively to complete the 2-fold dilution series.
- The undiluted standard serves as the high standard (1,000 pg/ml).
- Calibrator Diluent serves as the zero standard (0 pg/ml).

Storage/Stability

Unopened Kit

Store at $2 - 8^{\circ}\text{C}$. Do not use past kit expiration date.

Opened/ Reconstituted Reagents

Diluted Wash Buffer, Stop Solution, Calibrator Diluent 5, Calibrator Diluent 6, Assay Diluent 1F, Conjugate, Unmixed Color Reagent A and Unmixed Color Reagent B: May be stored for up to 1 month at $2 - 8^{\circ}\text{C}$, provided this is within the expiration date of the kit.

Standard: Discard the TNF- α stock solution and dilutions after 8 hours. Use a fresh standard for each assay.

Microplate Wells: Return unused wells to the foil pouch containing the desiccant pack, reseal along entire edge of zip-seal. May be stored for up to 1 month at $2 - 8^{\circ}\text{C}$, provided this is within the expiration date of the kit.

Procedure

Limitations of the Procedure

FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.

The kit should not be used beyond the expiration date on the kit label.

It is important that the Calibrator Diluent selected for the standard curve be consistent with the samples being assayed.

If samples generate values higher than the highest standard, dilute the samples with the appropriate Calibrator Diluent and repeat the assay.

As manufacturers we take great care to ensure that our products are suitable for use with all validated sample types, as designated in the product insert. However, it is possible that in some cases, high levels of interfering factors may cause unusual results. Any variation in standard diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding. Soluble receptors or other binding proteins present in biological samples do not necessarily interfere with the measurement of ligands in samples. However, until the factors have been tested in the assay, the possibility of interference cannot be excluded.

Assay Procedure

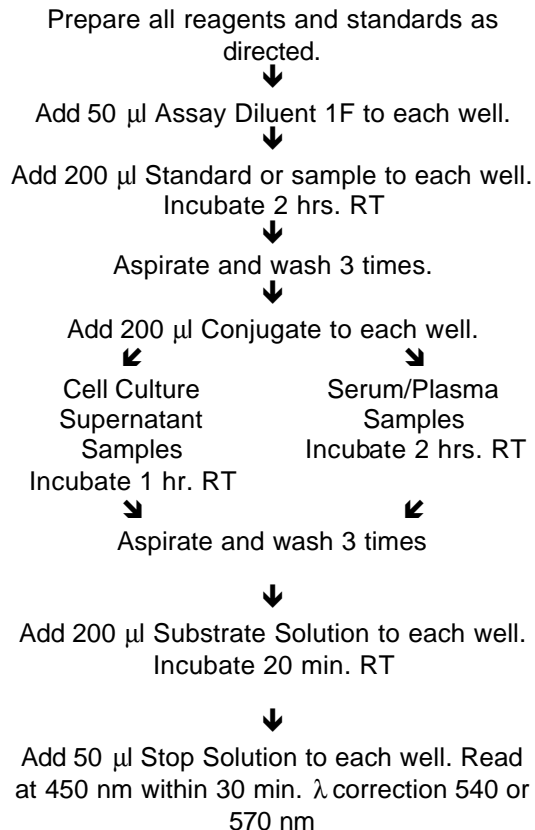
Bring all reagents and samples to room temperature before use. It is recommended that all samples and standards be assayed in duplicate.

1. Prepare all reagents and working standards as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, reseal.
3. Add 50 μ l of Assay Diluent 1F to each well. Assay Diluent 1F will have a precipitate present. Mix well before and during use.
4. Add 200 μ l of Standard or sample per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature. A plate layout is provided to record standards and samples assayed.
5. Aspirate each well and wash, repeating the process twice for a total of three washes. Wash by filling each well with Wash Buffer (400 μ l) using a squirt bottle, multi-channel pipette, manifold dispenser or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting.

Invert the plate and blot it against clean paper toweling.

6. Add 200 μ l of TNF- α Conjugate to each well. Cover with a new adhesive strip. For Serum/Plasma Samples: Incubate for 2 hours at room temperature. For Cell Culture Supernatant Samples: Incubate for 1 hour at room temperature.
7. Repeat the aspiration/wash as in step 5.
8. Add 200 μ l of Substrate Solution to each well. Incubate for 20 minutes at room temperature.
9. Add 50 μ l of Stop Solution to each well. If color change does not appear uniform, gently tap the plate to ensure thorough mixing.
10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

Assay Procedure Summary



Results

Calculation of Results

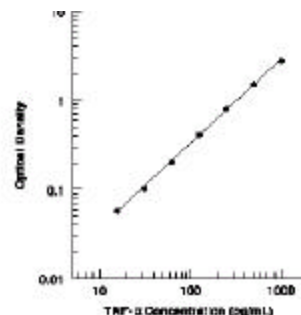
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density.

Plot the optical density for the standards versus the concentration of the standards and draw the best curve. The data can be linearized by using log/log paper and regression analysis may be applied to the log transformation.

To determine the TNF- α concentration of each sample, first find the absorbance value on the y-axis and extend a horizontal line to the standard curve. At the point of intersection, extend a vertical line to the x-axis and read the corresponding TNF- α concentration. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

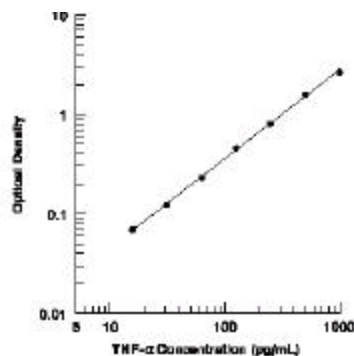
Product Profile

These standard curves are provided for demonstration only. A standard curve should be generated for each set of samples assayed.



Calibrator Diluent 5

(pg/mL)	O.D.	Average	Corrected
0	0.041 0.044 0.094	0.042	
15.6	0.107 0.145	0.100	0.058
31.2	0.145 0.241	0.145	0.103
62.5	0.248 0.451	0.244	0.202
125	0.462 0.847	0.456	0.414
250	0.853 1.552	0.850	0.808
500	1.529 2.791	1.540	1.498
1000	2.885	2.823	2.781



Calibrator Diluent 6

(pg/mL)	O.D.	Average	Corrected
0	0.061 0.063 0.129	0.062	
15.6	0.133 0.181	0.131	0.069
31.2	0.186 0.295	0.184	0.122
62.5	0.288 0.530	0.292	0.230
125	0.505 0.893	0.518	0.456
250	0.839 1.632	0.866	0.804
500	1.631 2.738	1.632	1.570
1000	2.686	2.712	2.650

Precision

Intra-assay Precision (Precision within an assay)

Three samples of known concentration were assayed twenty times on one plate to assess intra-assay precision.

Inter-assay Precision (Precision between assays)

Three samples of known concentration were assayed in twenty separate assays to assess inter-assay precision.

Serum/Plasma Assay						
	Intra-assay Precision			Inter-assay Precision		
	1	2	3	1	2	3
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (pg/ml)	48.1	317	587	45.8	301	587
Standard deviation	2.5	13.2	27.1	3.4	13.7	31.6
CV (%)	5.2	4.2	4.6	7.4	4.6	5.4

Cell Culture Supernatant Assay						
	Intra-assay Precision			Inter-assay Precision		
	1	2	3	1	2	3
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (pg/ml)	41.2	281	546	43.8	283	567
Standard deviation	2.2	12.5	27.3	3.0	24.7	41.5
CV (%)	5.3	4.4	5.0	6.8	8.7	7.3

Linearity

To assess linearity of the assay, five samples were spiked with high concentrations of TNF- α in various matrices and diluted with the appropriate Calibrator Diluent to produce samples with values within the dynamic range of the assay.

		Cell culture media	Serum	EDTA plasma	Heparin plasma	Citrate plasma
1:2	Average % of Expected	104	105	104	99	107
	Range (%)	95-108	100-113	100-108	91-103	101-114
1:4	Average % of Expected	105	102	105	100	106
	Range (%)	96-112	97-107	100-113	87-109	91-115
1:8	Average % of Expected	107	100	102	104	117
	Range (%)	94-121	96-109	93-107	95-110	109-126
1:16	Average % of Expected	101	97	102	108	109
	Range (%)	88-109	92-102	95-112	102-111	101-121

Sensitivity

The minimum detectable dose of TNF- α is typically less than 4.4 pg/ml.

The minimum detectable dose was determined by adding two standard deviations to the mean optical density value of twenty zero standard replicates and calculating the corresponding concentration.

Recovery

The recovery of TNF- α spiked to three different levels in five samples throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media	98	91 - 109%
Serum	107	94 - 119%
EDTA plasma	110	98 - 128%
Heparin plasma	101	89 - 115%
Citrate plasma	106	89 - 123%

Calibration

This immunoassay is calibrated against a highly purified *E. coli*-expressed recombinant human TNF- α . The NIBSC/WHO First International Standard 87/650 (recombinant human TNF- α expressed in *E. coli*) was evaluated in this kit. The dose response curve of this First International Standard parallels the assay's standard curve. To convert sample values obtained with the TNF- α ELISA kit to equivalent NIBSC 87/650 international units, use the equation below.

$$\text{NIBSC (87/650) equivalent value (IU/ml)} = 0.0344 \times \text{TNF-}\alpha \text{ ELISA kit value (pg/ml)}$$

Sample Values

Serum/Plasma –

Forty serum and plasma samples were evaluated for the presence of TNF- α in this assay. All samples measured less than the lowest standard, 15.6 pg/ml.

Cell culture supernatants –

Human peripheral blood mononuclear cells (1×10^6 cells/ml) were cultured in RPMI supplemented with 10% fetal calf serum, 50 μ M -mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μ g/ml streptomycin sulfate. Cells were stimulated for 1, 3, and 5 days. Aliquots of the culture supernatant were removed on days 1, 3, and 5 and assayed for levels of TNF- α .

Stimulant(s)	Day 0 (pg/ml)	Day 1 (pg/ml)	Day 3 (pg/ml)	Day 5 (pg/ml)
10 μ g/ml PHA	ND	3714	6740	7239
10 μ g/ml PHA + 10 ng/mL rhIL-2	ND	3326	5981	7205
50 ng/mL PMA	ND	1442	1274	2000
50 ng/mL LPS	ND	3045	1489	1233

ND = non-detectable

Specificity

This assay recognizes both natural and recombinant human TNF- α . The factors listed were prepared at 50 ng/mL in both Calibrator Diluent 5 and Calibrator Diluent 6, and assayed for cross reactivity. Preparations of the following factors at 50 ng/mL in a mid-range rhTNF- α control were assayed for interference. No significant cross-reactivity or interference was observed.

Human TNF- β , human soluble TNF RI and human soluble TNF RII recombinant proteins (factors related to or associated with TNF- α) were pre-incubated at 5, 10, and 50 ng/ml for up to 1 hour with sample prior to assaying. No significant cross-reactivity or interference was observed. No cross-reactivity or interference was observed with recombinant canine TNF- α at concentrations less than 10 ng/ml.

Other factors:

Recombinant human:

IL-1 α , IL-1 β , IL-1ra, IL-2, IL-3, IL-4, IL-5, IL-6, IL-6 sR, IL-7, IL-8, IL-9, IL-10, IL-11, ANG, β -ECGF, EGF, FGF acidic, FGF basic, FGF-4, G-CSF, GM-CSF, GRO α , IGF-I, IGF-II, IFN- γ , LIF, M-CSF, MCP-1, MIP-1 α ,

MIP-1 β , OSM, PDGF-AA, PDGF-AB, PDGF-BB, RANTES, SLPI, TGF- β 1, TGF- β 2, TGF- β 3, TGF- α

Recombinant mouse:

IL-1 α , IL-1 β , IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, EGF, GM-CSF, MIP-1 α , MIP-1 β , SCF, TNF- α

Other:

rrIL-1ra, bFGF acidic, bFGF basic, hPDGF, pPDGF, hTGF- β 1, pTGF- β 1, pTGF- β 1.2, pTGF- β 2, rcTGF- β 3, raTGF- β 5

Technical Hints

Substrate Solution should remain colorless until added to the plate. Substrate Solution should change from colorless to gradations of blue. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution.

Stop Solution should be added to the plate in the same order as the Substrate Solution.

When mixing or reconstituting protein solutions, always avoid foaming.

To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions.

Also, use separate reservoirs for each reagent.

To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.

References

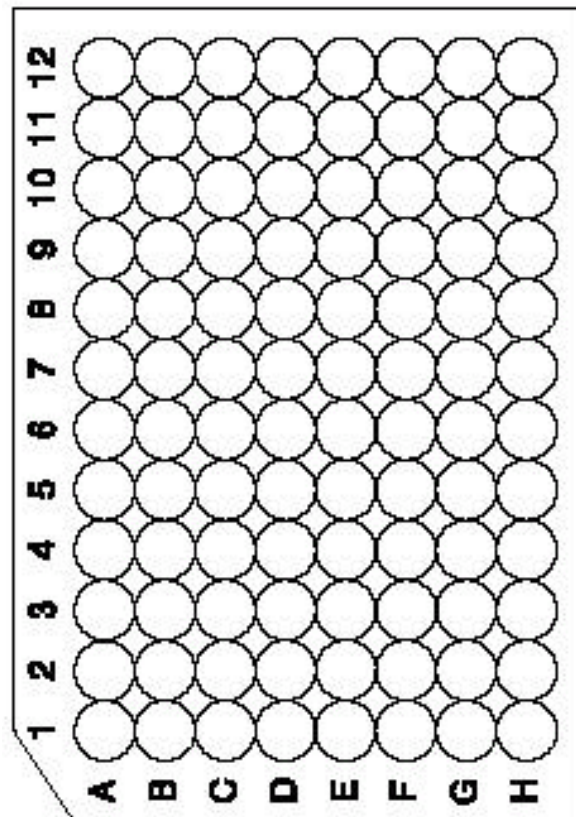
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lpg 6/00

PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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